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Assembly of a Protein-based Hydrogel as a Vehicle for In Situ Drug Delivery

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Assembly of a Protein-based Hydrogel as a Vehicle for *In Situ* Drug Delivery

By
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Dedication

I would like to dedicate this work to the family and friends who have shown me love over the past twenty-one years. To those who have supported me unconditionally throughout my successes and failures, you have made the hard times worth it and the good times exceptional. Without you all I wouldn't be where I am today, and I certainly would not be experiencing the happiness that I feel each day.

To my parents, thank you for believing in me and giving me the opportunity to receive an education and to be challenged on a daily basis. To the new friends I have made in college, the friends from high school that still support me, and the brothers of Kappa Alpha Order who have given me such a great college experience, thank you all. I certainly will continue doing my best, but I may never be able to repay all that you have each given me. While our times at Ole Miss shall too pass, my hope is that the next chapter will be even greater and more than we could ever have imagined. I love you all.

Acknowledgements

I would like to acknowledge Dr. Susan Pedigo for showing me a light in the space of college that is often dark and challenging. You've shown me that school is more than a grade and that the people worth celebrating are those that go the extra mile to connect with and care for others. Through both schoolwork and friendship you have made me feel loved and respected, and I can't thank you enough.

I would also like to acknowledge the Sally McDonnell Barksdale Honors College for funding this work and for giving me an outstanding educational experience.

ABSTRACT

Austin Heath Carroll: Assembly of a Protein-based Hydrogel as a Vehicle for *In Situ* Drug Delivery (Under the direction of Dr. Susan Pedigo)

The goal of this work is to develop a self-assembling, protein-based gel to deliver drugs at the site of chronic inflammation. There are two engineered protomers that comprise the gel; calmodulin- and M13- are their critical components. Numerous enzymes have been shown to undergo calcium-dependent regulation by calmodulin, an intracellular protein highly resilient to its chemical environment. One of these enzymes, myosin light chain kinase, contains a remarkable 26 residue portion known as the M13 peptide that has been demonstrated to bind to calmodulin in the presence of calcium through hydrophobic interactions. Because the extracellular space *in vivo* constitutes an environment rich in calcium, mixtures of engineered proteins with M13 (called PMSP) and calmodulin (called CMSP) will form a network. These engineered protein mixtures can be functionalized with drugs for delivery to the extracellular space at sites of chronic inflammation. This two component binding system can be tuned to modify the affinity and cooperativity with which self-assembly occurs, thus making it the perfect vehicle for *in situ* drug delivery. The purpose of the work reported here is to study the M13 peptide-containing protomer, PMSP. Since this is a novel construct, this thesis reports basic studies of the bacterial expression, solubility, interactions with calmodulin, and evidence that it forms a gel with CMSP. In order to obtain PMSP sufficient for hydrogel formation, we have bacterially expressed the protein. We have found PMSP to express poorly, with

yields increased upon expression from freshly transformed cells. Further, PMSP has limited solubility in aqueous solutions, requiring the addition of Guanidine HCl or DMSO to solubilize it. Once in solution, we found that the aqueous solubility of PMSP increased in the presence of free calmodulin implying that PMSP bound calmodulin. Secondly, PMSP appeared to bind to CMSP in the presence of calcium and to form a viscous solution. We are actively working to develop spectroscopic methods to support the apparent calmodulin-PMSP binding data. Finally, we will report our first attempts to measure the viscosity of the PMSP and CMSP mixture.

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LIST OF ABBREVIATIONS

AA	Amino Acid
°C	degrees Celsius
Ca^{2+}	Calcium Ion
CaM	Calmodulin
CAM K	CaM Kinase
CLS	Collagen Like Sequence
CMSP	Calmodulin-Modification-Sequence-Protein
C Terminus	Carboxyl Terminus
Da	Dalton
DMSO	Dimethyl Sulfoxide
DNA	Deoxynucleic Acid
E. coli	*Escherichia coli*
GI	Gastrointestinal
GND	Guanidine
HCl	Hydrogen Chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His Tag	Histidine Tag Purification
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_d	Disassociation Constant
NaCl	Sodium Chloride

MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MLCK	Myosin Light-Chain Kinase
mol	Mole
MW	Molecular Weight
N Terminus	Amino Terminus
NSAID	Nonsteroidal Anti-inflammatory Drug
pH	$-\text{Log}[H^+]$
PMSP	Peptide-Modification-Sequence-Protein
STD	Standard
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TX	Triton-X
UV-Vis	Ultraviolet-Visible Spectroscopy

INTRODUCTION

Inflammation is a critical response to injury or infection that provides the body with the ability to destroy pathogenic invaders and to repair damaged tissues [1]. During these acute periods, inflammation increases blood flow and recruits immune cells to the point of grievance leading to pain and swelling [2]. This inflammatory process is sequential with checkpoints in place to ensure that immune cell recruitment, microbe destruction, tissue liquefaction, and tissue repair occur in the correct order [3]. When this sequential process is disrupted, a holding pattern known as chronic inflammation occurs. While acute inflammation promotes tissue repair, chronic inflammation in diseases such as cancer, diabetes, cardiovascular disease, endometriosis, sciatic nerve pain and various autoimmune disorders [4] actually further damages tissues and oxidizes DNA [3]. Chronic inflammation also causes local acidification in the tissues [5] and the release of matrix metalloproteinases that remodel the extracellular space [6]. Treatment is necessary to circumvent and combat the destructive aspects of chronic inflammation.

The most common treatment for chronic inflammation is the use of non-steroidal anti-inflammatory drugs (NSAIDS) like aspirin, acetaminophen, and ibuprophen [7]. These drugs must be administered orally or intravenously, however, and are systemically distributed to all tissues. This systemic distribution leads to toxicity in the liver, kidneys, and GI tract. Over periods of chronic use, or as a result of extreme dosage, this collateral damage leads to organ failure [8]. The purpose of our system is thus to create an injectable, protein-based hydrogel capable of self-assembly *in vivo* that will bioconjugate with NSAIDs to deliver them to the extracellular space in a controlled manner. By

accomplishing this task, we will help circumvent the issue of NSAID toxicity and will provide a method for controlling chronic inflammation. As this system is developed, it is highly probable that the hydrogel will be useful in delivering other types of drugs as well.

Our protein-based hydrogel exploits the binding properties of two intracellular proteins: calmodulin (CaM) and M13. Calmodulin is an acidic, 148 residue amino acid protein that acts as a calcium-dependent regulator in a number of intracellular processes (**Figure 2a**) [9]. These processes range from signal transduction pathways involving the calmodulin-Ca^{2+} kinases (CaMK I-VI), AMPA receptor recycling in neurons [10], and smooth muscle contraction in an interaction with a specific portion of myosin light chain kinase (MLCK) [11]. This highly specific interaction of CaM with MLCK is the basis of the interactions between the protomers in our hydrogel **(Figure 1)**. As CaM binds M13, the previously unstructured M13 peptide assumes a helical shape, and the CaM protein becomes more compact [12].

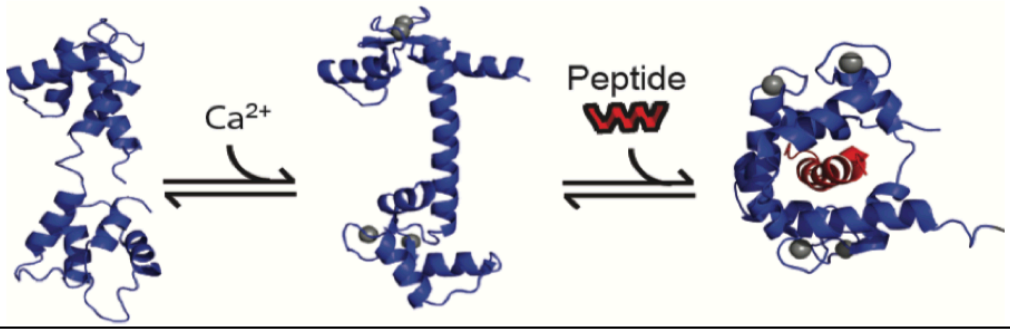

Figure 1. Calmodulin – Peptide Interaction: CaM (depicted in blue) binds tightly to a 26 amino acid portion of the enzyme myosin light chain kinase (depicted in red) when in the presence of calcium. When this binding occurs, the peptide develops a helical shape from a previously unstructured state while calmodulin becomes more compact.

MLCK is an enzyme present in smooth muscle that contains a 26 amino acid sequence known as M13 **(Figure 2b)**. This M13 sequence is critical for calcium dependent muscle contraction. As calcium enters the cellular cytoplasm, it binds to CaM (K_d = ~1 μM C-terminus, K_d = ~10 μM N-terminus), which then binds to the M13 portion of MLCK resulting in muscle contraction [13]. This binding occurs through hydrophobic interactions with very high affinity (K_d = < 10 nM) and is the basis for the formation of our hydrogel [14]. Because calcium is present in much greater concentrations in the extracellular space than inside the cytoplasm, our calmodulin-based component will be saturated with calcium and capable of binding the M13 component when injected into the extracellular space.

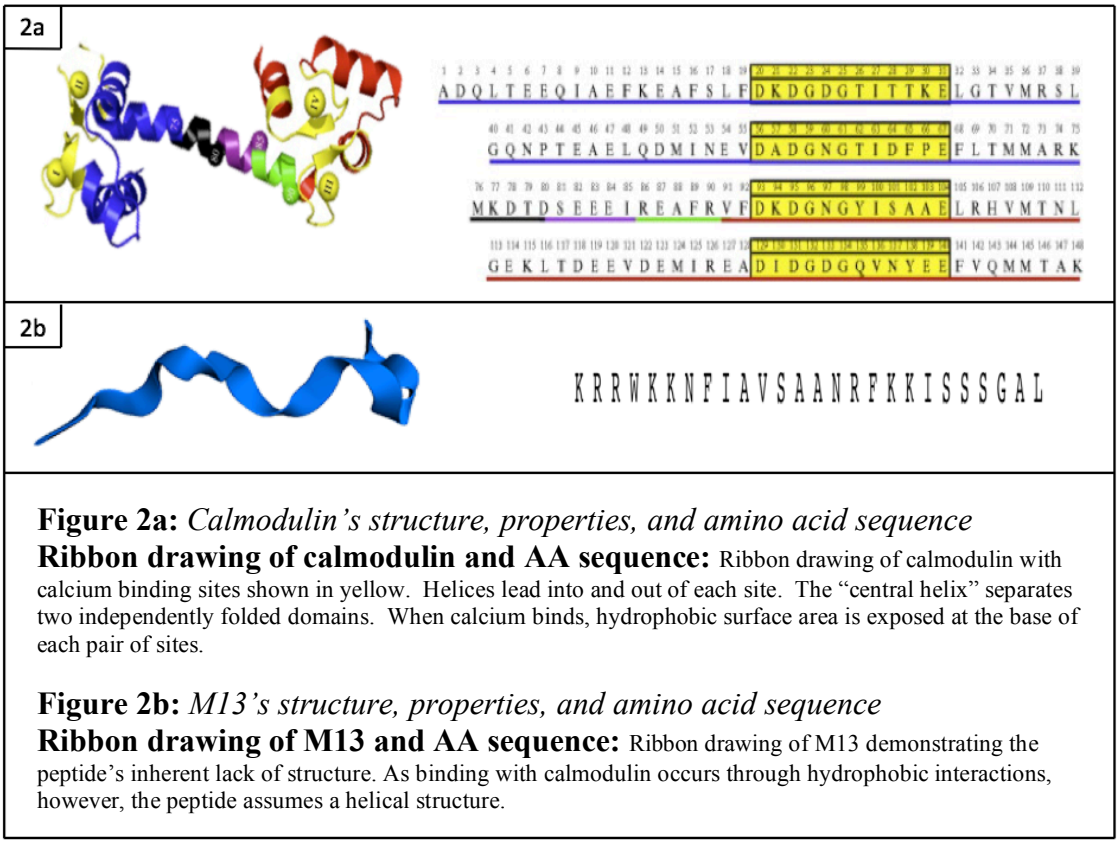

Figure 2a: *Calmodulin's structure, properties, and amino acid sequence*
Ribbon drawing of calmodulin and AA sequence: Ribbon drawing of calmodulin with calcium binding sites shown in yellow. Helices lead into and out of each site. The "central helix" separates two independently folded domains. When calcium binds, hydrophobic surface area is exposed at the base of each pair of sites.

Figure 2b: *M13's structure, properties, and amino acid sequence*
Ribbon drawing of M13 and AA sequence: Ribbon drawing of M13 demonstrating the peptide's inherent lack of structure. As binding with calmodulin occurs through hydrophobic interactions, however, the peptide assumes a helical structure.

PROTEIN DESIGN

Description of Protomers

Our experimental design makes use of two protomers derived from bacterially expressed, synthetic genes: one containing M13 and the other containing CaM. The M13-based protomer (PMSP for Peptide-Modification-Sequence-Protein) is the primary focus of this thesis and involves two repeats of M13 separated by "two cleavable modification sequences" (Figure 3). These modification sequences are similar to collagen and are sites for degradation by matrix metalloproteinases that are present in the extracellular space and increasingly secreted during times of inflammation [6]. PMSP also contains a 6-histidine sequence for Histag purification as well as a cysteine residue on the C-terminus that allows for the formation of a disulfide linkage to another PMSP in oxidizing conditions. The two repeats of M13 within the protomer provide high affinity binding sites for the calmodulin-based protomer when in the presence of calcium. The calmodulin-based protomer (CMSP) is nearly identical to PMSP and contains two sequences of CaM separated by two collagen-like sequences. Like the PMSP component, it also contains a 6-histidine sequence on the N-terminus for purification and a terminal cysteine on the C-terminus for creating a disulfide linkage with identical protomers (**Figures 3a** and **3b**).

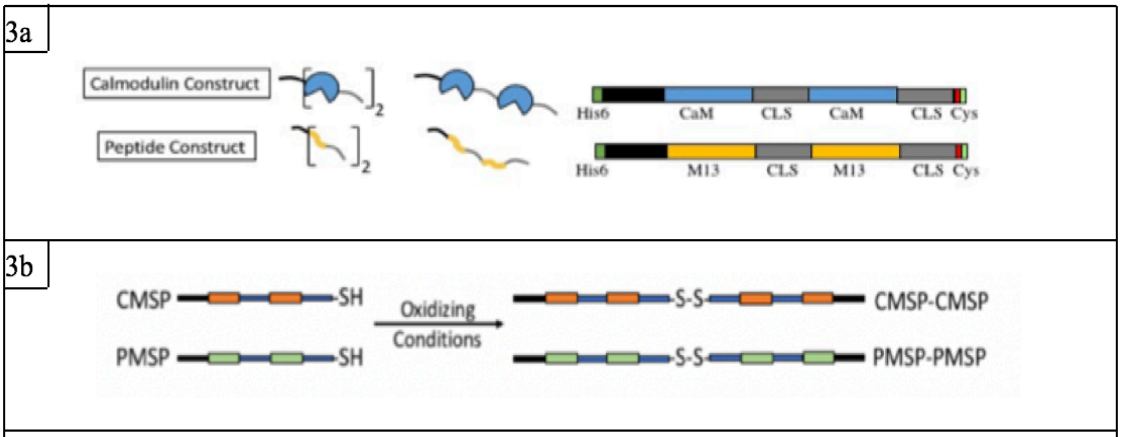

Figure 3a: *CMSP and PMSP Constructs*
Depictions of CMSP and PMSP: Each protomer contains a Histag sequence depicted in green and black, two repeats of CaM or M13, two repeats of a collagen—like sequence (CLS), and a terminal cysteine.
Figure 3b: *Formation of Protomer Dimers*
Depiction of Dimer Formation
Under oxidizing conditions, the C-terminus cysteine residues allow the protomers to form disulfide linkages. This allows for the formation of four binding sites on each of the constructs.

Hydrogel Design

Our design makes use of the inherent properties of a hydrogel, which is a 3D network of crosslinked polymers that retain high amounts of water and demonstrate elasticity and strength similar to bodily tissues [15]. Through the use of biomaterials capable of binding in response to extracellular calcium, our hydrogel will self-assemble and respond to inflammation. Through a collaborative project with the Watkins lab, we will bioconjugate NSAIDs to the calmodulin protomer through ester linkages, and the gel will respond to the increased acidity in the extracellular space during times of inflammation. As the pH decreases, the ester linkages will be hydrolyzed and the drug will be increasingly delivered. Degradation of the gel by proteases and consequent drug release will also be further accelerated by the increased release of matrix metalloproteinases during times of inflammation. As needed, the composition, size, and

drug dosage of the hydrogel can be modified, making it the perfect vehicle for *in situ* drug delivery.

Structure of the Thesis

Since PMSP is a completely novel protein, there is no precedence for its properties. As we worked with this engineered protein, we encountered a number of unexpected results that then drove the next steps. We present this "odyssey" as it unfolded, and while the story is still developing, this thesis documents the path so far.

SCOPE OF WORK

To obtain PMSP, we transformed BL21DE3 Rosetta cells with a pET30 plasmid containing our gene that confers kanamycin resistance (**Figure 4a**). By growing our cells on agar plates spiked with kanamycin, we were able to successfully confirm that transformation occurred. IPTG, a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon, was then used to induce bacterial expression of our protein. After expression, the cells were centrifuged, sonicated and centrifuged once more (**Figure 4b**). An acrylamide gel was then used to demonstrate that PMSP was present in the post-induction pellet and in the sonicated whole cell but not in the post-induction supernatant (**Figure 5a**). This important piece of data revealed that PMSP is not soluble in aqueous solutions. This forced us to recover it from the pellet. Thus, the pellet was treated with Triton-X, a non-ionic surfactant capable of permeabilizing cell membranes. Three washes were carried out with an initial wash using 10% Triton-X and

two subsequent washes using 1% Triton-X. Each wash was followed by centrifugation. Samples were taken before and after each wash, and after centrifugation for an SDS-acrylamide gel (**Figure 5b**), which demonstrated that the protein remained in the pellet after the 10 % TX and 1% TX washes.

In order to solubilize the pellet, we tried several strategies. First, in previous work from our lab, Christopher Fox showed that the PMSP was not adequately solubilized in concentrated urea. Thus, 6 M Guanidine HCl/20 mM Tris/ 140 mM NaCl/pH 7.4 was added to the pellet. After significant vortexing, the pellet was dislodged and mixed with the aqueous solvent. At this point, a large gelatinous material descended to the bottom of the conical (**Figure 6b**), and we speculated that it could be DNA based on its appearance. This gel was mechanically removed, washed with distilled water three times, and was then sonicated for 30 seconds and centrifuged for 30 minutes at 3000 rpm. An SDS-acrylamide gel was run to evaluate the protein in the supernatant and gel portions of the gelatinous material (**Figure 5c**). A subsequent UV spectrum was also taken of the supernatant but due to scattering of light by insoluble material, no clear signal could be obtained.

The Triton-X, Guanidine-treated supernatant was then taken into 1 mL aliquots, centrifuged for 8 minutes and placed on a Histag column equilibrated with Binding Buffer + 6 M Guanidine. Wash buffers and elution buffers also containing 6 M Guanidine were used to maintain protein solubility through the entire separation. Subsequently, an acrylamide gel and UV-Vis spectrum revealed that PCLP had been successfully purified for the first time (**Figure 5d** and **Figure 6a**), but that it had low solubility in aqueous buffers. There were two elution fractions with PMSP with

concentrations of ~ 40 μM and 100 μM. Studies described in this thesis were conducted with the 40 μM fraction.

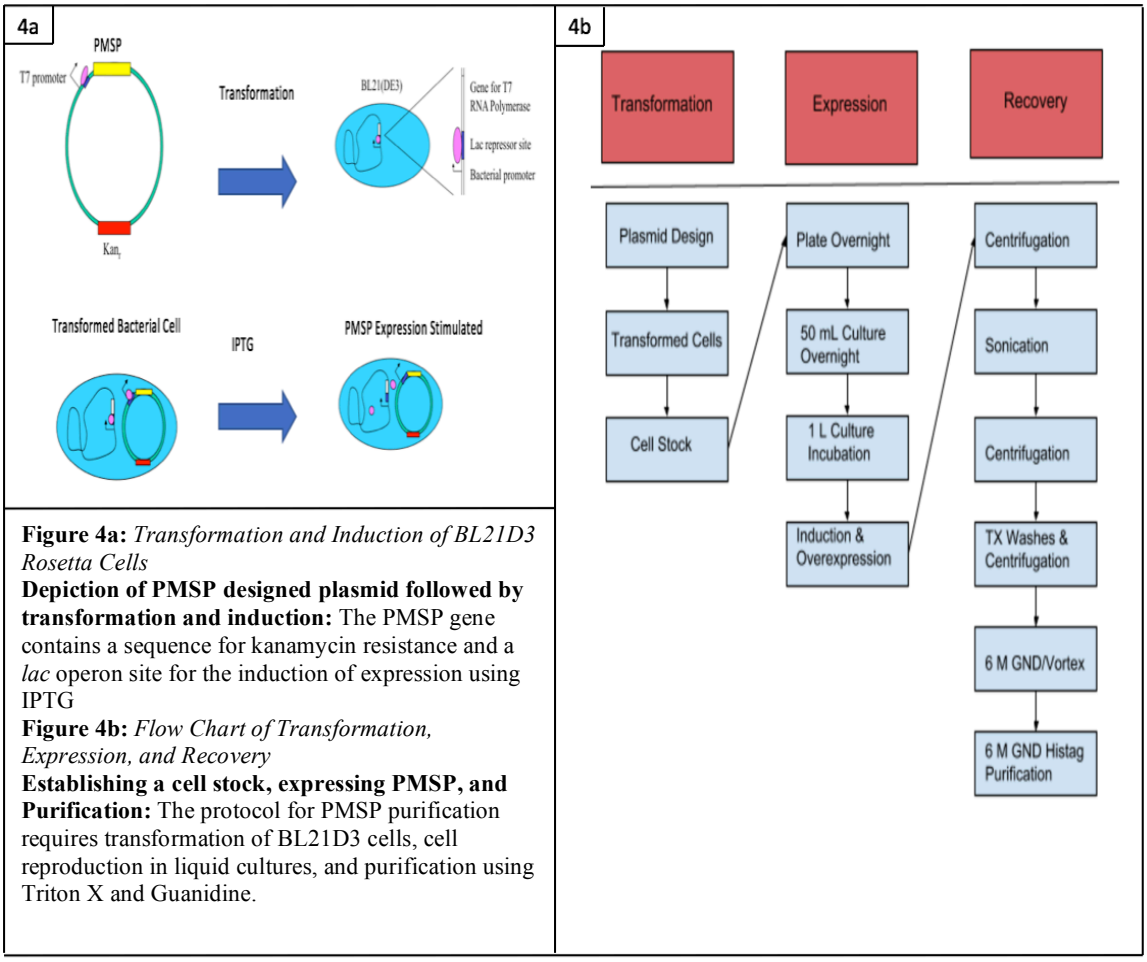

Figure 4a: *Transformation and Induction of BL21D3 Rosetta Cells*
Depiction of PMSP designed plasmid followed by transformation and induction: The PMSP gene contains a sequence for kanamycin resistance and a *lac* operon site for the induction of expression using IPTG
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Establishing a cell stock, expressing PMSP, and Purification: The protocol for PMSP purification requires transformation of BL21D3 cells, cell reproduction in liquid cultures, and purification using Triton X and Guanidine.

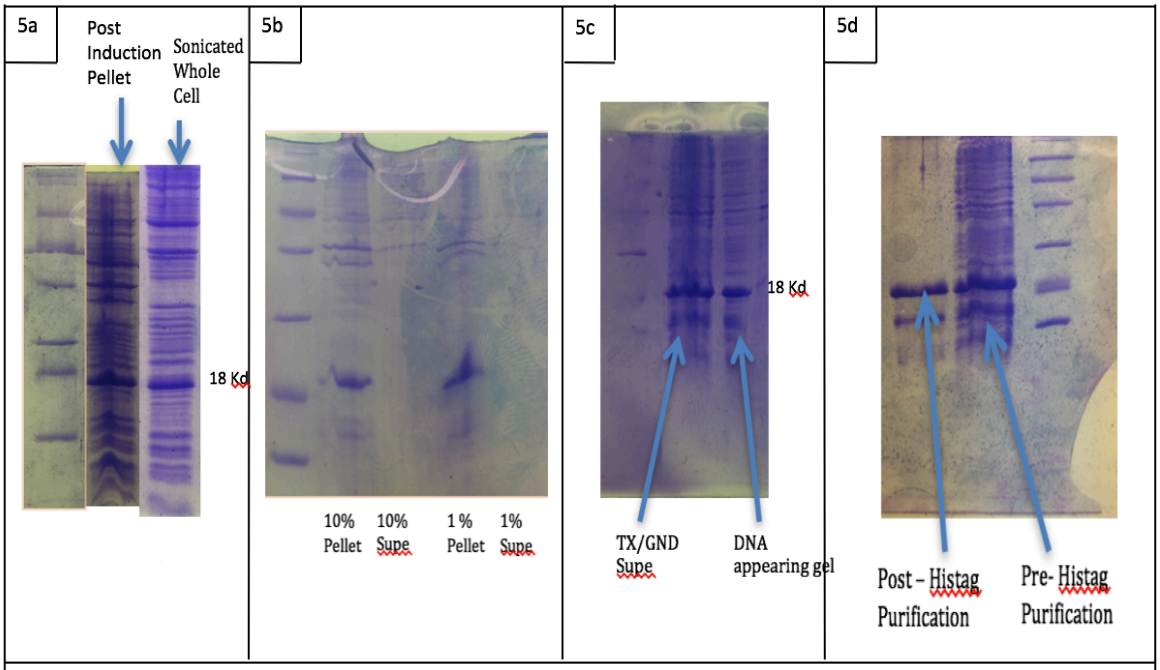

Figure 5a: *Acrylamide gel demonstrating initial expression and sonification of PMSP*
Post induction sample and sonicated whole cell: After induction with IPTG, the gel revealed that PMSP was present at 18 kD in both the post induction sample and the sonicated whole cell sample. This indicates that PMSP is not soluble in aqueous solution
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Guanidine Treatment and gelatinous material analysis: Treatment with guanidine allowed the PMSP to move into solution as shown in the gel. The gelatinous material was also demonstrated to contain PMSP.
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Histag Chromatography Purification: Histag purification using binding, wash, and elution buffers containing 6 M Guanidine led to the purification of PMSP. Material visualized below 18 kD shows truncated products.

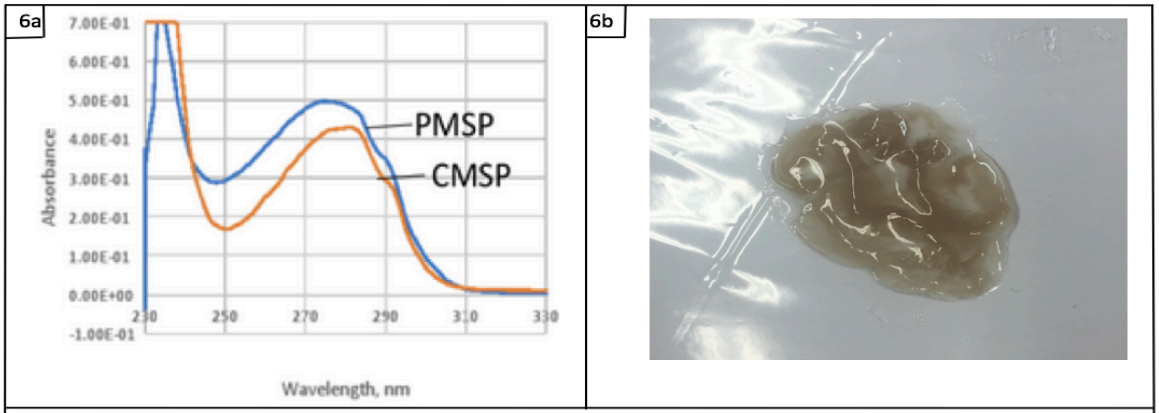

Figure 6a: *UV-Vis Spectrum Demonstrating the Purification of PMSP*
Figure 6b: *Gelatinous material that was Mechanically Removed from PMSP after the addition of 6 M Guanidine*

Solubility Tests

Through the expression and purification of PMSP, we have determined it is largely insoluble in aqueous solution. It was thus necessary to carry out several solubility tests to determine feasible solvents for mixtures with CMSP. The first test included dialyzing a portion of PMSP stock out of aqueous guanidine and into pure DMSO. Through this transition, the PMSP remained fully soluble, and it was determined that DMSO was a feasible solvent for working with our protein.

The second test consisted of dialyzing a portion of PMSP into 100 mL solutions of aqueous SEC buffer consisting of HEPES and NaCl with gradually decreasing amounts of guanidine. The solutions ranged from 6 M, 4.5 M, 3 M, 1.5 M, and 0.5 M guanidine. Each solution was dialyzed for approximately an hour. The PMSP remained fully soluble until the transition from 2 M to 0.5 M guanidine. At this point, the protein formed a heavy, white precipitate. Interestingly, no gelatinous material reformed.

Next, we hypothesized that the addition of hydrophilic CaM would increase the aqueous solubility of PMSP. To this end, 3 mg of dry CaM and concentrated aqueous calcium to a concentration of 1 mM were added to the precipitated solution. These two additions did not bring the precipitated PMSP back into solution. This mixture was dialyzed into 200 mL of 2 M guanidine/20 mM HEPES/ 140 mM NaCl/pH 7.4 (SEC buffer) for one hour and the protein returned into solution. After this, the solution was dialyzed into 200 mL of pure SEC buffer for 2 hours. This time, the protein remained fully soluble in the aqueous SEC solution (**Figure 7**). This indicates that 1) PMSP binds to CaM in the presence of calcium, and that 2) the binding of CaM to PMSP increases the

solubility of PMSP.

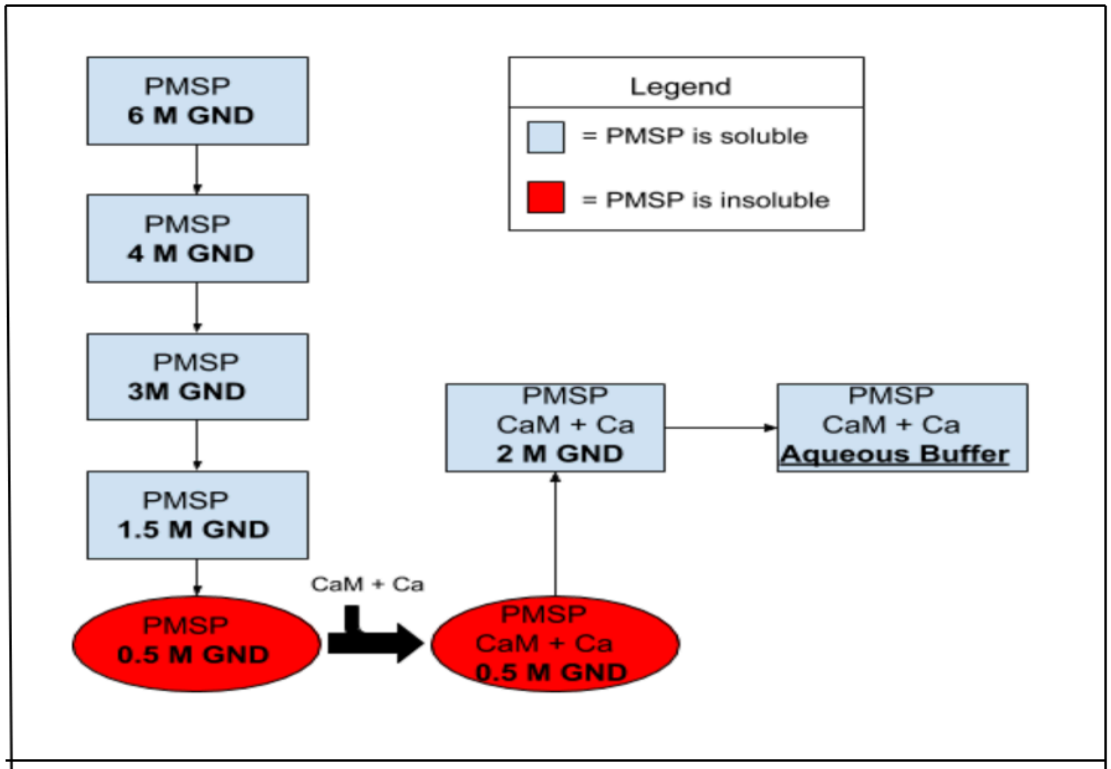

Figure 7: *Solubility Schematic of PMSP in SEC Buffer with varying Concentrations of Guanidine* **Decreasing Guanidine Decreases Solubility but adding CaM and Ca^{2+} Increases Solubility:** As the concentration of guanidine is decreased, solubility decreases. At 0.5 M guanidine, PMSP precipitates out of solution. As demonstrated, when CaM and Ca^{2+} are added, PMSP remains fully soluble in aqueous SEC buffer. This indicates that PMSP is binding CaM.

After testing PMSP alone and in the presence of CaM, we tested the solubility of PMSP in the presence of the CMSP protomer. This was accomplished by adding 5 mg of dry CMSP to 1.5 mL of 40 μM PMSP in 6 M guanidine and dialyzing it against aqueous SEC buffer. After an hour, an extremely white, heavy precipitate formed and the solution was dialyzed into a 2 M guanidine solution. This allowed the protein to return to solution. Finally, the solution was dialyzed against pure DMSO, and the CMSP/PMSP mixture remained soluble.

Formation of a Hydrogel

In order to form our first hydrogel, we concentrated the CMSP/PMSP mixture in DMSO to 113 µM PMSP and 225 µM CMSP and added calcium to a final concentration of approximately 50 mM. Within five minutes, the solution became turbid and took upon the appearance of a viscous gel (**Figure 8c**).

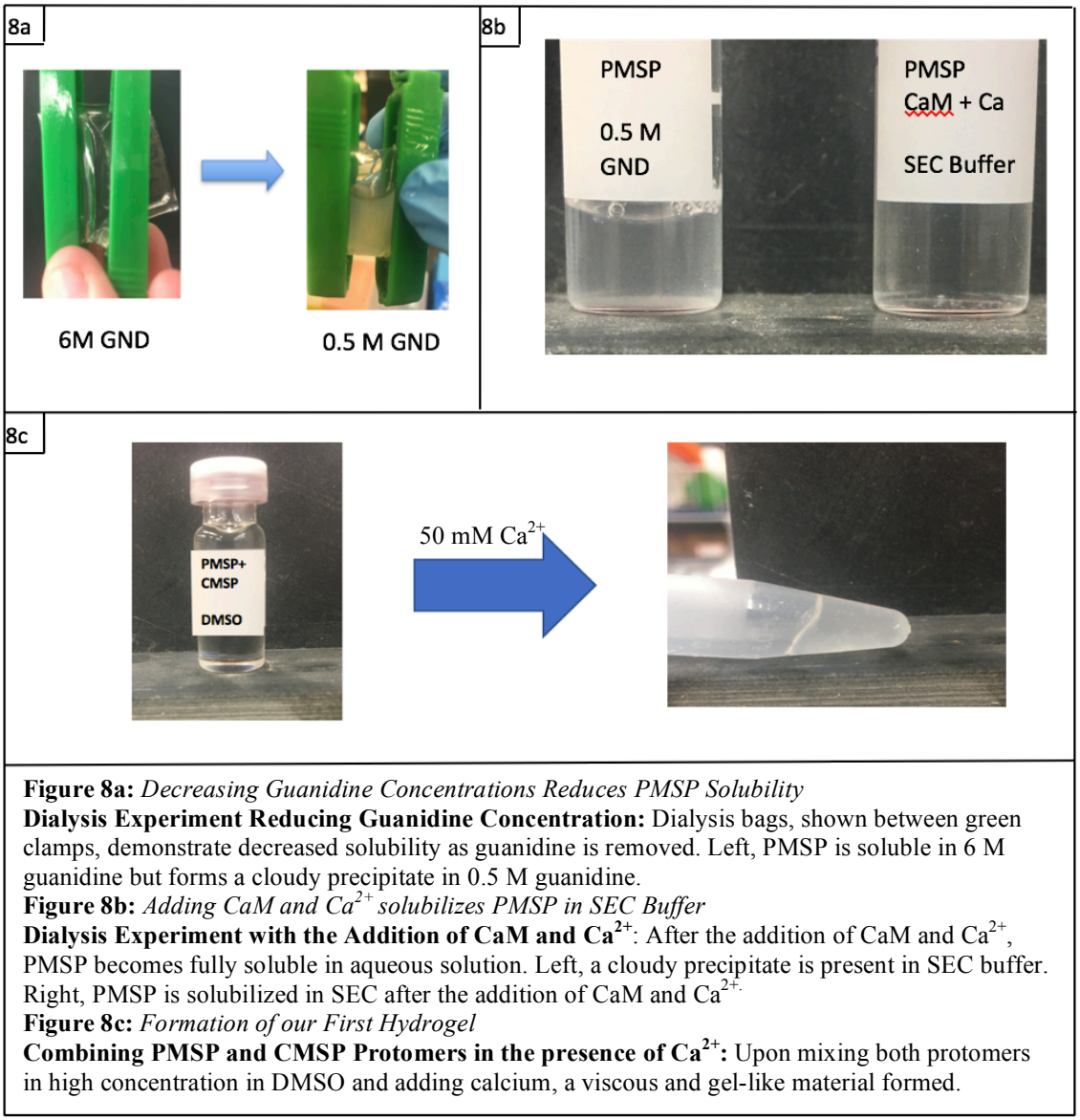

Figure 8a: *Decreasing Guanidine Concentrations Reduces PMSP Solubility*
Dialysis Experiment Reducing Guanidine Concentration: Dialysis bags, shown between green clamps, demonstrate decreased solubility as guanidine is removed. Left, PMSP is soluble in 6 M guanidine but forms a cloudy precipitate in 0.5 M guanidine.
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Dialysis Experiment with the Addition of CaM and Ca^{2+}: After the addition of CaM and Ca^{2+}, PMSP becomes fully soluble in aqueous solution. Left, a cloudy precipitate is present in SEC buffer. Right, PMSP is solubilized in SEC after the addition of CaM and Ca^{2+}.
Figure 8c: *Formation of our First Hydrogel*
Combining PMSP and CMSP Protomers in the presence of Ca^{2+}: Upon mixing both protomers in high concentration in DMSO and adding calcium, a viscous and gel-like material formed.

Synthetic Peptide Alternative

As a possible alternative for circumventing the solubility issue of the PMSP protomer, we also designed a short 48 amino acid peptide with the following sequence: CGGSS**KRRWKKNFIAVSAANRFKKISSSGAL**GSREGPQGIWGQERSGC (The 26 residue M13 sequence is in bold and the collagenase cleavage sequence is underlined. Cysteines are at the termini). To synthesize the peptide, we used a CEM peptide synthesizer in the lab of Dr. Saumen Chakraborty with the help of Dr. Chakraborty and Suchitra Mitra. This peptide contains the full M13 sequence and only a single collagen-like modification sequence to increase solubility. Cysteines are present at each end to allow linkages of the peptide segments into protomers with a diversity of lengths and geometries. Just after synthesis, the peptide was cleaved from the bead, and a MALDI-TOF mass was obtained of the peptide product. The mass spectrum demonstrated an envelope of products ranging from a mass of 4.8 kD to 5.7 kD (**Figure 9a**), a range that spans the expected molecular weight (5.1 kD). We are not clear as to what the envelope of products indicates, although we have speculated that truncated products and impurities are present in the sample. Upon analysis with an acrylamide gel using samples with BME reducing loading buffer in DMSO and SEC solutions and a sample with non-reducing loading buffer in SEC solution, bands were visualized at approximately 10 kD **(Figure 9b)**. This size is the expected weight of peptide dimers, although it is unclear why polymerization occurred regardless of whether or not reducing agent was present in the loading buffer, and that polymerization would make only dimers. Through the use of an Innovagen peptide properties calculator (https://pepcalc.com/), it was revealed that the peptide contains a significant number of basic residues that could be preventing it from

properly migrating in the electrophoretic field of an acrylamide gel and could thus explain why all three bands from **Figure 9b** were visualized at 10 kD. As for solubility, the peptide was found to be fairly soluble in aqueous solutions. This gives it potential for use in the drug delivery system, and further work is necessary to functionalize the peptide and determine how it reacts in the presence of CMSP and calcium.

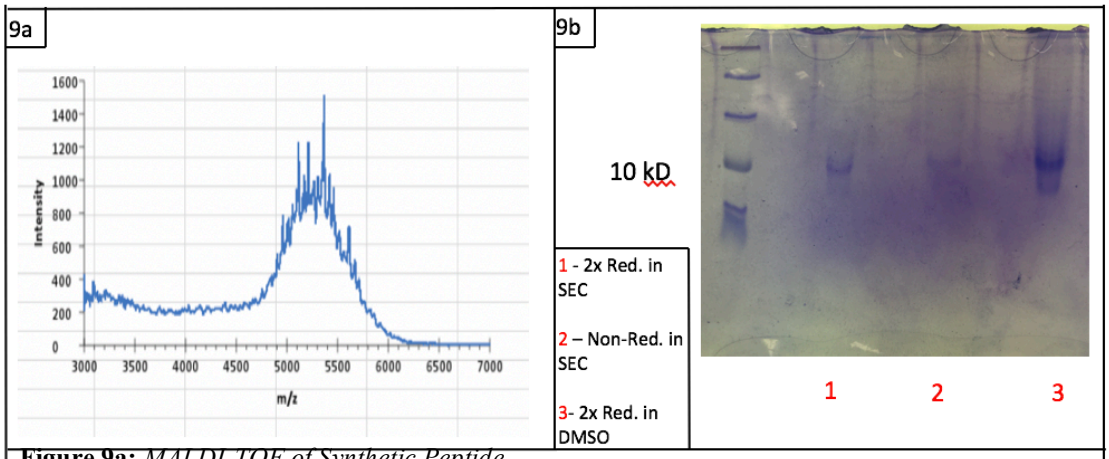

Figure 9a: *MALDI-TOF of Synthetic Peptide*
Envelope of Products Revealed by MALDI Analysis: The crude sample from the peptide sample revealed an envelope of products ranging from 4.8 kD to 5.7 kD. The expected weight of the peptide was within the envelope at 5.1 kD.
Figure 9b: *Acrylamide Gel of Synthetic Peptide*
Acrylamide Gel with Reducing and Non-Reducing Loading Buffer: Samples in DMSO and SEC solutions with BME reducing load were visualized as bands at 10 kD. A sample in SEC solution with a non-reducing load was also visualized at 10 kD.

DISCUSSION AND CONCLUSION

The experiments described above indicate that our engineered PMSP construct has low aqueous solubility, an attribute that introduces a challenge that subverts its role as one of two components in an aqueous hydrogel. Below we speculate on a number of aspects of the experiments reported above and of the structure and function of PMSP in an attempt to better understand the challenges that this protein has presented. A better understanding of these challenges will help us to best determine the conditions for its use in our nascent biomaterial and will allow us to design a second generation PMSP protomer.

Bacterial Expression of PMSP

There are distinct advantages to using a bacterial expression system for making our PMSP protomer. These expression systems are inexpensive, produce large quantities, and provide an inexpensive method for re-engineering the gene to improve the properties of the protomer. Our current system has several properties that we do not entirely understand. First, in our lab, we typically store transformed expression cells as glycerol stocks at -80 °C. The CMSP expression cells are stored in this way, yet we have found that expression of PMSP is very poor in subsequent attempts after the first expression. We have successfully produced PMSP from the transformed cell stocks, but in low abundance. We have also tried an alternative cell line, and found the same phenomenon: after storage for one to two weeks, expression levels have dropped. Thus, to obtain decent yield upon expression, we must use freshly transformed cells. Interestingly, this is

not required of the CMSP expression cells even though the CMSP gene is identical to the PMSP gene except for the peptide/CaM components. There is no secondary structure formation that we expect in the mRNA. As of yet, we don't have an explanation for this complication in the expression of PMSP.

PMSP's Insolubility

From previous experience using a 100 μM M13 stock in our lab, we have demonstrated that the M13 peptide is quite soluble in aqueous solutions. The PMSP protomer was thus designed with the belief that it would be fully soluble. Personal communication with Dr. Madeline Shea (University of Iowa, Department of Biochemistry) informed us to expect that M13 would interact with negatively charged phospholipids in the cell membrane during bacterial expression due to the fact that it contains a significant number of positively-charged, basic residues. For this reason, we expected that PMSP would be in the pellet fraction after sonication. We reasoned, however, that treatment with Triton-X would dissolve and remove the cell membrane lipid component, and increase the solubility of PMSP. As discussed above, PMSP remained insoluble after the Triton-X washes.

Because PMSP remained in the pellet after the washes, we have been forced to investigate other potential issues that could be causing the lack of solubility. One promising possibility remains the number of collagen-like modification sequences within each PMSP molecule. First, the collagen-like sequences contain hydrophobic amino acids, no charged amino acids (GPQGIWGQ), and only two H-bond donor/acceptors. From this inherent hydrophobicity, it is likely that the collagen-like sequences are playing

a role in PMSP's insolubility in aqueous solutions. The fact that the synthetic peptide with only a single collagen-like sequence demonstrated an increased solubility over PMSP supports this conclusion. This argument also raises the question of why the CMSP protomer is so soluble. One explanation is that the large hydrophilic CaM components are far larger than the collagen-like sequences and provide increased solubility. The importance of CaM in increasing the solubility of the CMSP protomer is further supported by the fact the PMSP protomer is only soluble in aqueous solution when in the presence of added CaM, as discussed above.

Second, it is possible that the collagen-like sequences within multiple PMSP protomers are aggregating with one another to form the triple helix structure that composes collagen polymers. A brief review of the literature suggests it takes a minimum of 36 amino acids of collagen repeats (twelve repeats of the 3- amino acid collagen sequence) for the formation of collagen triple helixes [16]. Because our material only contains 8 amino acids in each collagen-like segment, we speculate that formation of a full collagen triple helix is not occurring.

The Role of DMSO

In order to work with PMSP, we needed a solvent that would solubilize our protomer. It was also important to find a solvent for biomaterial formation that is compatible with medical applications and that would be safe when administered within the body. We thus chose DMSO, although we are working to increase the aqueous solubility of the peptide construct in hopes that we will be able to work in aqueous solutions in the future.

DMSO is an amphipathic molecule with both a highly polar sulfonyl bond, and two non-polar methyl groups [17]. It has a half-life of 16 hours within humans and can be administered in a number of different ways for medical applications [18]. The most common routes of administration include topical administration to assist in drug delivery and via intravesical instillation in women with interstitial cystitis [19]. It is important to note that DMSO is capable of increasing the flexibility of the phospholipid bilayer in cell membranes and increasing the number of water pores in the membrane [20]. This property makes it particularly useful as a vehicle for drug delivery as it easily penetrates skin and other bodily barriers. Fortunately, this membrane penetration does not cause irreversible damage, and DMSO has been shown to have low toxicity in humans [17]. This, in concert with the fact that DMSO itself has been directly administered as a medication, makes it an applicable solvent for our drug delivery system.

To ensure that DMSO would not prohibitively disrupt PMSP and CMSP binding, we conducted a brief literature review on the effect that DMSO has on general protein structure. The literature suggests that DMSO increasingly disrupts protein secondary structure as DMSO concentration increases. This occurs primarily through the disruption of protein hydrogen bonding networks [21]. In pure DMSO, it was found that a majority of proteins are almost completely unfolded and tend to form aggregates. Our data with CMSP and PMSP forming a turbid material in DMSO suggests that the two protomers are still cable of binding when in the presence of extraordinarily high concentrations of calcium. We speculate that the high levels of calcium drive the maintenance of secondary structure within the highly resilient CMSP and allow it to bind PMSP, which is already unstructured. It will be necessary to conduct further binding studies between CaM and

M13 and between PMSP and CMSP in DMSO to determine the optimum conditions for binding. In theory, these speculations should be testable, but since DMSO has a large absorbance in the region of the spectrum in which secondary structures absorb light, we cannot use instrumentation routinely available to us. We are actively working on using fluorescence to monitor the environment of the tryptophan residues as a function of CaM binding and solvent composition.

Implications and Direction for Future Research

At this time, the CMSP component is fully functional and behaves as expected (soluble and demonstrates calcium-dependent M13 binding). It will thus be important to continue to study PMSP in DMSO, work to increase its aqueous solubility, and to characterize the formation of gels. One promising alternative remains to design a gene that contains only a single collagen-like modification sequence to increase the hydrophilic/hydrophobic ratio of the protein. In addition, it will be important to further investigate the gelatinous material that was removed in **Figure 6b** and to confirm the increase in viscosity observed in **Figure 8c** through the use of rheometry. Furthermore, it will be necessary to assess the susceptibility of the components to degradation by proteases that will be present in the extracellular matrix. In conclusion, both protomers have shown promise as a means to create a novel *in-situ* delivery system.

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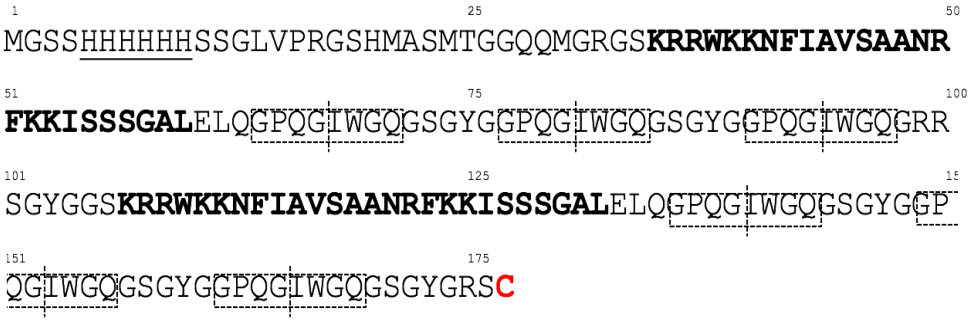
APPENDIX



Appendix 1: ***Full Amino Acid Sequence of PMSP Protomer****:* This schematic provides the full AA sequence for our protomer. The 6 histidine histag sequence is underlined. In bold, the 28 AA sequence for the M13 peptide. The collagen-like modification sequences are shown in boxes. Finally, the terminal cysteine is shown in red.